JOURNAL OF VIROLOGY, Dec. 1989, p. 5399–5404
0022-538X/89/125399-06$02.00/0

Vol. 63, No. 12

The Secretory Core Protein of Human Hepatitis B Virus Is Expressed on the Cell Surface

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Received 12 June 1989/Accepted 12 August 1989

Human hepatitis B virus (HBV) causes acute and chronic liver disease, which can result in tumor formation. An as yet unexplained phenomenon is that virus elimination usually correlates with the development of antibodies directed against the HBeAg, a secretory HBV core gene product which can be detected in the serum of infected patients. Expression of HBeAg in a human hepatoma cell line by using recombinant vaccinia viruses revealed that the HBeAg is not only secreted from HBeAg-producing cells but also incorporated into the outer cell membrane. No membrane-expressed core gene product could be detected when the cytoplasmic core protein (HBcAg) was expressed. Immune sera from patients who developed anti-HBe antibodies efficiently recognized the membrane-bound HBeAg, suggesting that surface-expressed HBeAg can serve as a target for an antibody-mediated elimination of HBV-infected cells.

Human hepatitis B virus (HBV) is the prototype member of the hepadnavirus family, a small group of primarily hepatotropic enveloped DNA viruses which replicate by reverse transcription (10). A characteristic feature of HBV infections is that a certain proportion of infected individuals do not clear the virus and thus since HBV is not cytopathic, become permanent producers of virus or viral proteins (12, 13). These chronic carriers represent a major health problem, since they serve as a source for new infections and, in addition, are at high risk of developing liver cirrhosis or liver cancer (25).

The factors which are crucial for whether the infection becomes chronic are unknown. That the status of the immune system is of major importance is strongly suggested by the fact that newborns infected with HBV almost invariably develop chronic hepatitis, whereas more than 95% of infected adults clear the virus (12, 13). However, tolerance is not always permanent, and, even after years of chronic infection, virus can be eliminated spontaneously (7). The most important marker indicating virus clearance in chronic as well as in acute cases is the development of antibodies directed against the HBeAg, a secretory form of the viral capsid protein which can be detected in the serum of infected individuals (19). Why the development of such antibodies, which are directed against a protein that is not a component of the viral envelope, correlates with virus elimination is unknown.

Here we show that the HBeAg is not only secreted from HBeAg-producing cells but also incorporated into the outer cell membrane. Immune sera from patients who developed anti-HBe antibodies during infection efficiently recognize this membrane-bound HBeAg. These results suggest that surface-expressed HBeAg can serve as a target for the antibody-mediated elimination of HBV-infected cells. The temporary or permanent inability of certain patients to produce such antibodies might be an important factor leading to the development of chronic HBV infection.

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MATERIALS AND METHODS

Construction of recombinant vaccinia viruses. For construction of E-Ag VAC, a *Hin*dIII-*Bam*HI fragment containing HBV sequences from NT3091-1004 was excised from plasmid pMH3/3091 (15) and cloned into an M13 vector. The C-AUG was converted into an AUA by site-directed mutagenesis with a single-mismatch 18-mer oligonucleotide (sequence: TTGGGGCATA*GACATCGA) by following a standard protocol (34). Mutants were detected by selective hybridization with the mutagenizing oligonucleotide. After plaque purification, the base exchange was confirmed by sequence analysis and the fragment was recloned into a vaccinia virus expression vector (kindly provided by A. von Brunn, University of Heidelberg), which allows the expression of foreign proteins under the control of the 7.5-kilodalton early/late promoter (the 7.5K early/late promoter). For construction of the recombinant encoding the wild-type HBeAg, the unaltered *Hin*dIII-*Bam*HI fragment was used. For construction of C-Ag VAC, which lacks the first nucleotide of the pre-C AUG, a *Hin*dIII-*Bam*HI fragment was excised from plasmid pMH3/3097 (15) and directly cloned into the expression vector. Production and selection of vaccinia virus recombinants were performed as described previously (16, 18). Recombinants expressing the respective HBV core gene products were detected by infecting HU TK^- 143 cells (6) with plaque-purified virus and screening the cell lysates for HBeAg and HBcAg by a commercial radioimmunoassay (see below). For bulk cultures and titer determinations of the recombinant viruses, the same cell line was used.

Infection of HepG2 cells. HepG2 cells (2) (10^6 cells grown in a 10-cm^2 dish with 2 ml of Dulbecco modified minimal essential medium containing 10% fetal calf serum at 5% CO_2) were infected with recombinant or wild-type vaccinia virus at a multiplicity of infection of 10, as determined by the titer in the HU TK^- 143 cells, in 0.7 ml of serum-free medium. After 1 h at 37°C, the inoculum was removed and 2 ml of fresh medium with 10% fetal calf serum was added.

Determination of HBV core gene products by radioimmunoassay. For determination of HBV core gene products in infected cells, the HBe (rDNA) diagnostic kit from Abbot Laboratories, Wiesbaden, Federal Republic of Germany,

which detects both HBcAg and HBeAg, was used. The medium was removed, and the cells were lysed in phosphate-buffered saline (PBS) with 1% Triton X-100 (1 ml for a 10-cm^2 dish). For radioimmunoassay 200 μl of cleared cell lysate or 200 μl of medium was used.

Detection of HBV core gene products by Western blotting and indirect immunofluorescence. Immunoprecipitation and Western immunoblotting were performed essentially as described previously (27, 28), with a polyclonal rabbit antiserum raised against denatured recombinant HBcAg (anti-HBc/e antiserum). This antiserum recognizes both the HBcAg and the HBeAg. To test for membrane-expressed HBV core gene products, cells grown and infected in 10-cm^2 dishes as described above were chilled on ice, washed with ice-cold PBS, and incubated for 45 min on ice with the anti-HBc/e antiserum diluted 1:100 in PBS–2% bovine serum albumin–0.1% NaN_3. After washing with PBS–2% bovine serum albumin–0.1% NaN_3 (30 min on ice) and with PBS, bound antibody was detected by incubating the cells for 30 min on ice with 0.7 ml of goat anti-rabbit antiserum labeled with fluorescein isothiocyanate (FITC; Dianova GmbH, Hamburg, Federal Republic of Germany) diluted 1:40 in PBS–2% bovine serum albumin–0.1% NaN_3. Washing conditions were as above. Staining with human antisera was done in the same way, except that an FITC-labeled goat anti-human antiserum was used. Fluorescence was examined by using a Fluovert inverted fluorescence microscope (E. Leitz Inc., Rockleigh, N.J.) at a magnification of 1:100.

To detect cytoplasmic core gene products, cells were fixed directly in the tissue culture dishes with 2 ml of 2% paraformaldehyde in PBS for 30 min at room temperature. After being washed with PBS and subjected to a 10-min incubation with 50 mM NH_4Cl–PBS, the cells were permeabilized by treatment with 0.1% Triton-X 100 in PBS for 4 min at room temperature and stained with antibody as described above.

Preparation of cells for flow cytometry. Cells were stained without fixation in the tissue culture dishes with the anti-HBc/e antiserum and the goat anti-rabbit FITC antiserum as described above. After being stained, the cells were gently washed off the plates and propidium iodide (10 μg/ml) was added. Analysis was performed with a FACStar Plus cell sorter (Becton Dickinson and Co., Paramus, N.J.) with 150 mW of 488-nm light (Lexel 95-4; Polytec GmbH, Karlsruhe, Federal Republic of Germany). Computer gating was used on propidium iodide (red) fluorescence to exclude dead cells, and forward and 90° light scatter were used to exclude debris and aggregates.

RESULTS

Construction of recombinant vaccinia viruses. Hepatitis B viruses express the core protein in a secretory and a nonsecretory form. This is due to the optional usage of a signal sequence encoded by a small open reading frame (pre-C region) located immediately upstream of the core gene (15, 22, 28). In the case of HBV, translation initiation at the pre-C AUG (nucleotide 3096) leads to the synthesis of the secretory form (HBeAg), whereas synthesis of the cytoplasmic core protein (HBcAg) initiates at the C-AUG (nucleotide 1; Fig. 1, top). As a first step to examining the role of these proteins in the antiviral immune response, recombinant vaccinia viruses expressing only either the HBeAg or the HBcAg were constructed (Fig. 1, top). The vaccinia virus expression system was selected, since these viruses allow the expression of foreign proteins in a variety of cell types which then can be used in immunological experiments, e.g., as target cells for cytotoxicity assays (20). The recombinant designated E-Ag VAC can code for only the HBeAg, since the core gene sequence introduced downstream from the vaccinia virus promoter (7.5K early/late promoter) starts with the pre-C AUG (nucleotide 3096) and, in addition, the C-AUG has been converted to an AUA by site-directed mutagenesis. The recombinant called C-Ag VAC can code for only the HBcAg, since the introduced core gene sequence starts with nucleotide 3097 and therefore lacks the first nucleotide of the pre-C AUG.

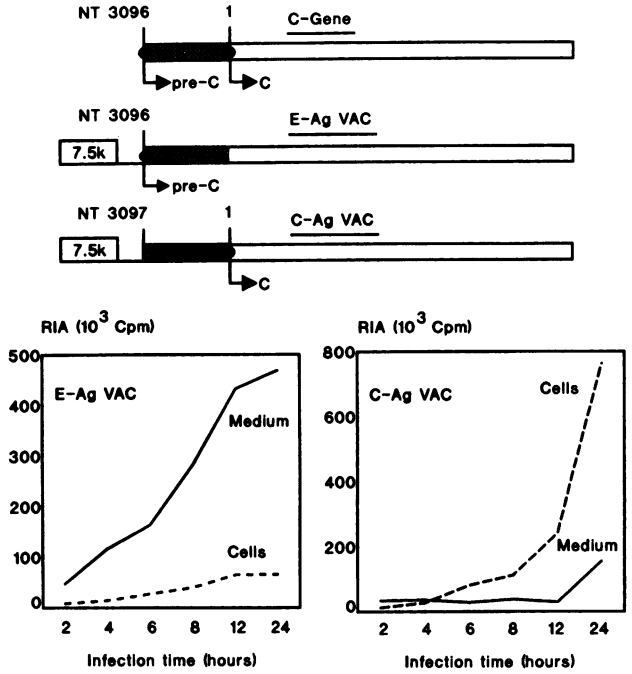

FIG. 1. Expression of HBeAg and HBcAg in HepG2 cells by using recombinant vaccinia viruses. The upper panel shows a schematic representation of the HBV pre-C/C-gene, which encodes both HBcAg and HBeAg. The pre-C region is shaded. To express each of these proteins independently, recombinant vaccinia viruses were constructed which expressed C-gene sequences coding either for the HBeAg (E-Ag VAC) or the HBcAg (C-Ag VAC) under control of the 7.5K early/late promoter. To analyze antigen synthesis, HepG2 cells were infected with E-Ag VAC or C-Ag VAC, and after various time, the total amount of core gene products present in medium or cell lysate were determined by using a commercial radioimmunoassay (RIA; lower panel).

Expression of HBeAg and HBcAg in HepG2 cells. The recombinant vaccinia viruses were then used to infect HepG2 cells, a well-differentiated human hepatoma cell line (2). This cell line was selected because it represents one of the few lines which produce complete HBV particles upon transfection with viral DNA (1, 31) and therefore should process newly synthesized HBV core gene products in the same way as authentic liver cells do. As demonstrated by the result of the radioimmunoassay shown in the lower panel of Fig. 1, HepG2 cells infected with E-Ag VAC, which encodes the HBeAg, produced large amounts of a secretory core gene product, whereas the core gene product encoded by C-Ag VAC (HBcAg) remained within the cells. In fact, HBcAg could be detected in the medium only 24 h after infection, a time point when some cell lysis occurs.

For further analysis of the core gene products synthesized by the HepG2 cells upon infection with E-Ag VAC and C-Ag VAC, cells were lysed in PBS–1% Triton X-100 at various

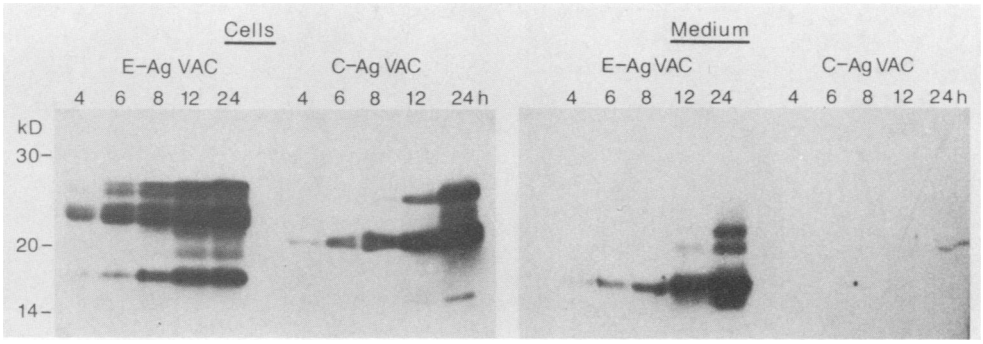

FIG. 2. Western blot analysis of HBV core gene products from HepG2 cells infected with recombinant vaccinia viruses. hepG2 cells grown in 10-cm^2 dishes were infected with either E-Ag VAC or C-Ag VAC, and after various times, the cells were lysed in PBS–1% Triton X-100. Core gene products were recovered from the cell lysate and the tissue culture medium by immunoprecipitation and separated on a 12.5% polyacrylamide gel. After Western transfer, the nitrocellulose filter was incubated with a polyclonal rabbit antiserum which recognizes both HBcAg and HBeAg, and specifically bound antibody was detected with ^{125}I-labeled protein A.

time points after infection. The cell lysates were then subjected to immunoprecipitation with a polyclonal anti-HBc/e antiserum, and the immunoprecipitates thus obtained were analyzed by Western blotting. After infection with E-Ag VAC, at least five different core gene products with sizes between 17 and 27 kilodaltons (kDa) could be detected in the cell lysates (Fig. 2, left). These proteins might represent HBeAg precursors as well as by-products like the N-terminal processed and probably further modified cytoplasmic form of the HBeAg, which has been described recently (11, 29). Only one of these proteins, the 17-kDa HBeAg, was efficiently secreted from the cells into the medium (Fig. 2, right). As expected, infection with C-Ag VAC resulted in the production of the HBcAg, which is about 21 kDa (Fig. 2, left). However, besides the HBcAg, another core gene product, of about 26 kDa could also be detected. Since the HBV core gene sequence cloned into C-Ag VAC cannot encode a protein of that size, this 26-kDa protein might be due to a modification of the HBcAg, the nature of which is unknown. As was already suggested by the result of the radioimmunoassay, only minute amounts of HBcAg were released into the medium, despite the very high rate of core

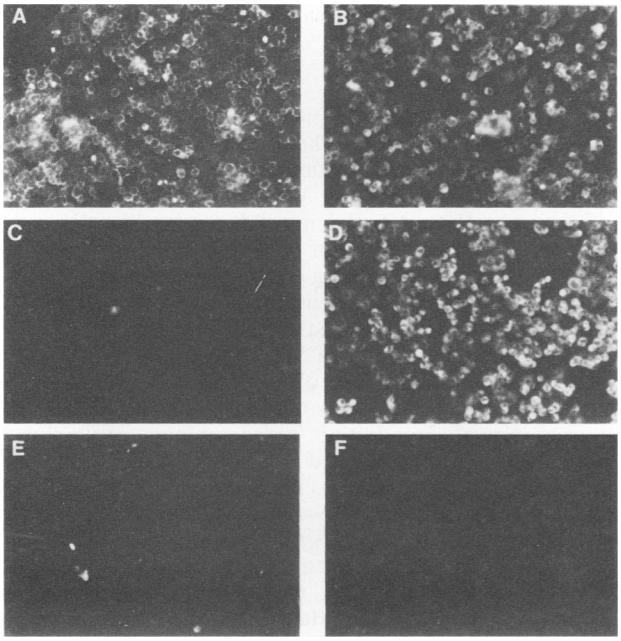

FIG. 3. Detection of membrane-expressed core gene products by indirect immunofluorescence. HepG2 cells grown in 10-cm^2 dishes were infected with either E-Ag VAC (A and B), C-Ag VAC (C and D), or wild-type vaccinia virus (E and F). After 12 h, the cells were either directly incubated with a polyclonal antiserum specific for both HBeAg and HBcAg (panels A, C, and E) or fixed with paraformaldehyde and permeabilized with Triton-X100 before being stained (panels B, D, and F). Bound antibody was detected with affinity-purified FITC-labeled goat anti-rabbit immunoglobulin.

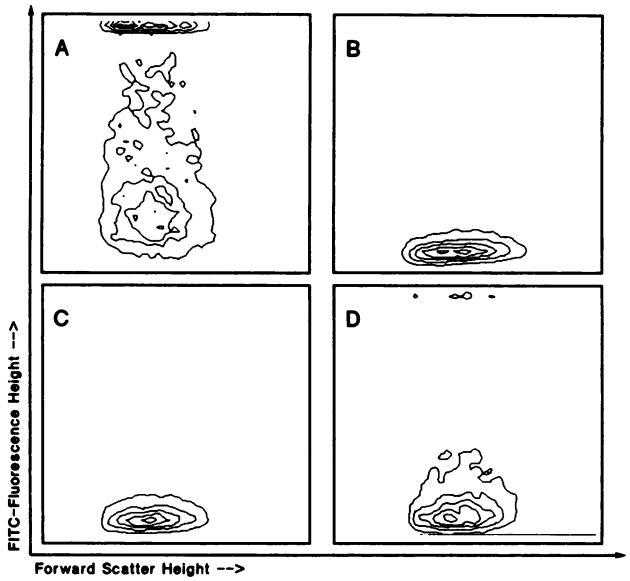

FIG. 4. Detection of membrane-expressed core gene products by flow cytometry. HepG2 cells were infected for 12 h with E-Ag VAC (A), C-Ag VAC (B), or wild-type vaccinia virus (C) as described in the legend to Fig. 1. To test for the possibility of passive HBeAg adsorption, uninfected cells were grown for 12 h in 2 ml of medium obtained from a HepG2 culture which had been infected for 24 h with E-Ag VAC (D). Virus had been removed from this medium by ultracentrifugation for 30 min at 435,000 × g prior to use. Data are presented as contour plots (20% intervals, 1% threshold). The y axis represents fluorescein fluorescence; the x axis represents forward (narrow-angle) light scatter.

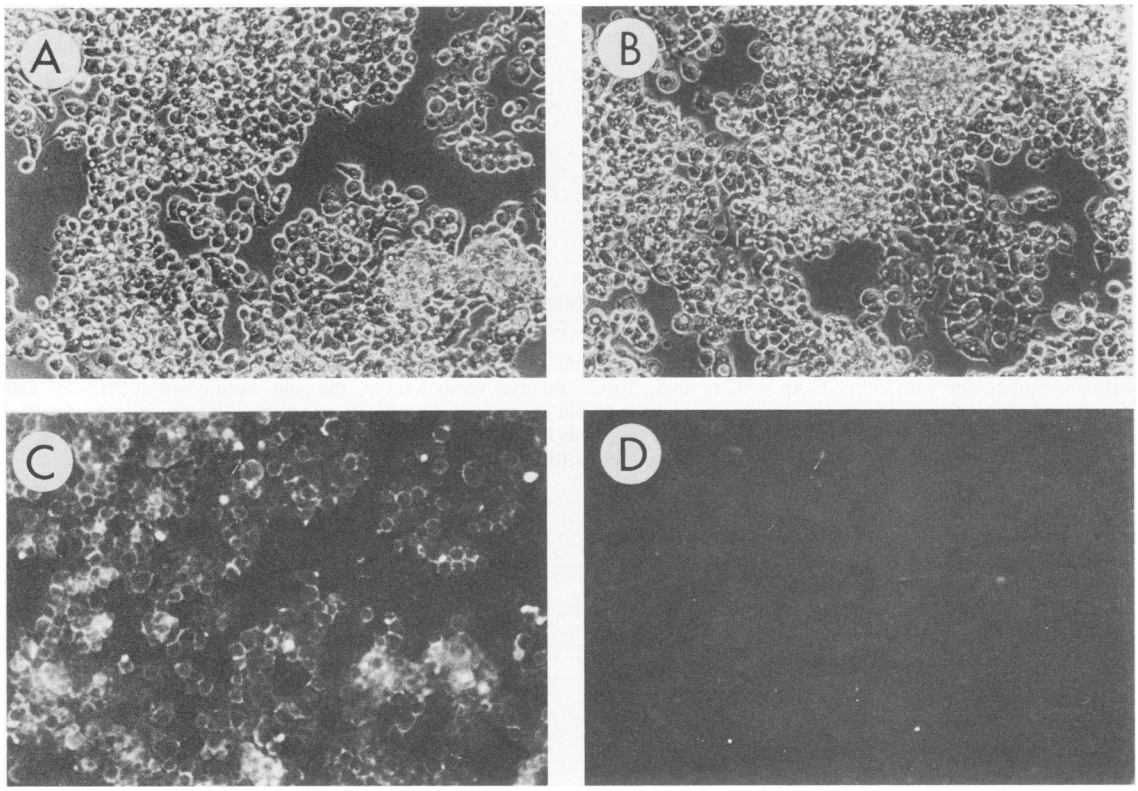

FIG. 5. Reaction of membrane-expressed HBeAg with a human anti-HBeAg positive immune serum. HepG2 cells were infected with either E-Ag VAC (left) or C-Ag VAC (right). After 12 h, the cells were directly incubated with a human immune serum containing anti-HBe antibodies (diluted 1:100), and specifically bound antibody was detected with an FITC-labeled goat anti-human immunoglobulin. Upper panel, Bright-field image; lower panel, FITC fluorescence.

protein biosynthesis (Fig. 2, right). Therefore, in contrast to a recent report in which it was speculated that a distinct pathway for core particle secretion might exist (14), there appears to be no mechanism for core particle secretion except cell lysis.

Detection of a membrane-expressed core gene product. During the last few years, several reports have been published in which it was proposed that expression of the HBcAg is not restricted to the cytoplasm but that this protein could also be incorporated into the outer cell membrane (7, 8, 23). However, the mechanism by which this protein might be transported to the cell surface remained obscure. To test, whether an HBV core gene product can reach the cell surface, HepG2 cells were infected with either E-Ag VAC, C-Ag VAC, or wild-type vaccinia virus. After 12 h, the cells were chilled on ice, directly incubated with a polyclonal rabbit antiserum specific for both HBeAg and HBcAg, and then incubated with a fluorescence-labeled anti-rabbit antibody. To control the expression efficiency, parallel samples were fixed and stained with antibody after permeabilization of the cells with Triton X-100. Nonpermeabilized cells infected with E-Ag VAC exhibited a bright membrane fluorescence, whereas cell infected with C-Ag VAC or wild-type vaccinia virus did not (Fig. 3). That large amounts of HBcAg are expressed at this time point is demonstrated by the C-Ag VAC-infected cells treated with detergent prior to staining.

To substantiate this finding, HepG2 cells infected and stained as described above were examined by flow cytometry (Fig. 4). The results completely agreed with the immunofluorescence analysis. The HBeAg-expressing cells showed a strong membrane fluorescence (Fig. 4A), whereas the cells infected with C-Ag VAC (Fig. 4B) or wild-type vaccinia virus (Fig. 4C) did not. Moreover, if cells were grown in conditioned medium containing large amounts of HBeAg, no significant staining could be observed (Fig. 4D), excluding the possibility of passive adsorption of secreted HBeAg. If the human fibroblast cell line HU TK^- 143, which is used for selecting vaccinia virus recombinants (6), was used, the same results were obtained, showing that membrane expression of HBeAg is not restricted to HepG2 cells (data not shown). In addition, a recombinant containing a C-gene sequence which started with the pre-C AUG but still contained the C-AUG behaved the same as E-Ag VAC, thus demonstrating that the single-amino-acid exchange present in the HBeAg encoded by E-Ag VAC owing to the mutation in the C-AUG was not important for membrane expression (data not shown).

The membrane-expressed HBeAg is efficiently recognized by human immune sera. To test whether the membrane-expressed HBeAg is recognized by human antisera, HepG2 cells infected with E-Ag VAC or C-Ag VAC were incubated with sera from patients who developed anti-HBe antibodies during infection. Bound antibodies were then detected with a fluorescence-labeled anti-human antibody. A typical result of such an analysis is shown in Fig. 5: cells infected with E-Ag VAC reacted strongly with the human serum, whereas cells infected with C-Ag VAC did not. This clearly demon-

strates that anti-HBe antibodies which develop during natural HBV infection can bind efficiently to the membrane-expressed HBeAg. In a preliminary study, five of nine patient serum samples containing anti-HBe antibodies reacted with the membrane-expressed HBeAg.

DISCUSSION

From the results described above, we conclude that the HBeAg is an unusual secretory protein, since it is not only secreted from HBeAg-producing cells but also incorporated into the outer cell membrane. To our knowledge, to date there is only one other example of a viral nucleocapsid protein which also exists in a membrane-bound and a secreted form, namely, the *gag* protein of murine leukemia viruses (9, 17, 24). How the HBeAg reaches the cell membrane is unknown. As shown recently by in vitro translation studies, transport of the HBeAg across the endoplasmic reticulum membrane can be aborted before translocation is complete, probably resulting in a transmembrane configuration (5). This might be due to the highly positively charged C terminus of the HBeAg precursor, which is clipped off during HBeAg biosynthesis (32), probably at a late stage (29). If complete translocation indeed requires the removal of this hydrophilic sequence, escape from proteolytic processing might result in the formation of a transmembraneous HBeAg, which then is transported to the cell surface.

What is the significance of a membrane-bound HBV core gene product? In previous studies it has been suggested that a surface-expressed HBcAg might be the target for tissue damage by cytotoxic T cells (7, 8, 23). However, the data presented here clearly show that the cell-surface-expressed HBV nucleocapsid protein is not derived from the cytoplasmic HBcAg but rather from the secretory HBeAg. Besides providing a rationale for how a HBV core gene product can reach the cell surface, this distinction is important because the HBcAg and the HBeAg (and possibly also the membrane-expressed HBeAg) differ significantly with respect to their serologic reactivity (26). Moreover, it is now generally accepted that membrane expression of native viral antigens is not important for cytotoxic T-cell recognition, since these cells recognize intracellularly generated peptide fragments complexed to major histocompatibility complex antigens (3, 4, 33). Surface expression of HBeAg, however, must have consequences in patients who develop a humoral immune response against this protein. That such antibodies can, in fact, mediate the elimination of virus-infected cells is strongly suggested by the findings that (i) seroconversion from HBeAg to anti-HBeAg usually correlates with virus clearance (7, 13) and (ii) HBV infection was delayed or prevented in chimpanzees after passive immunization with anti-HBeAg (but not with anti-HBcAg) (30) and active immunization with bacterially expressed HBV core gene products with HBeAg antigenicity (21).

ACKNOWLEDGMENTS

We thank A. von Brunn for help with vaccinia virus recombination, T. Prospero for flow cytometry, L. Theilmann for patient sera, F. Bles for excellent technical assistance, and N. Schek for critically reading the manuscript.

This work was supported by the Bundesministerium für Forschung und Technologie and the Deutsche Forschungsgemeinschaft.

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